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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
A61K 37/02, 37/22, 37/48, 39/35, 39/395

A1

(11) International Publication Number: **WO 94/21281**

(43) International Publication Date: 29 September 1994 (29.09.94)

(21) International Application Number: PCT/US94/03102

(22) International Filing Date: 22 March 1994 (22.03.94)

(30) Priority Data:
08/035,640 23 March 1993 (23.03.93) US

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(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: POLYMER-POLYPEPTIDE COMPOSITION AND METHOD

(57) Abstract

An improvement in a method for therapeutic treatment is disclosed. The treatment involves administering a polymer-polypeptide composition to a subject prior to administration of the polypeptide in free form. The polymer-polypeptide composition includes the polypeptide and polyethyleneglycol chains, surrounding, but not covalently attached to, the polypeptide.

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POLYMER-POLYPEPTIDE COMPOSITION AND METHOD

1. Field of the Invention

The present invention relates to an improvement in a method for treating a subject by parenteral administration of an immunogenic polypeptide. This invention also relates to a composition utilized for parenteral administration of the polypeptide.

2. References

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3. Background of the Invention

A variety of medical conditions can be treated by parenteral administration of antibodies or enzyme polypeptides. For example, monoclonal or polyclonal antibodies against different epitopes of a patient's CD4+ cells are administered alone, or in combination with other immunosuppressive compounds, for treatment of rheumatoid arthritis and other autoimmune diseases, or for the suppression of graft/host reactions or transplantation rejection. For treating tumors, anti-tumor antibodies may be coupled to toxins or radionucleotides, or to chemotherapeutic drugs for targeting these compounds to tumor cells (Sehon, 1992).

Among therapeutic enzymes, streptokinase is commonly administered for the treatment or prevention of blood clotting and thrombus formation. Tissue plasminogen activator is also used to prevent thrombus formation (Cherng).

In many cases, these therapeutic polypeptides are derived from nonhuman sources, or otherwise may include regions which are able to provoke an immune response. Repeated administration of such polypeptides can result in undesired side effects, such as serum sickness, anaphylactic symptoms, and/or liver complications.

To reduce immunological complications from treatment with foreign antibodies, a chimeric antibody constructed to have human constant regions and mouse variable regions has been

proposed (Greiner). However, the antibody may still provoke an immune response against the variable regions.

In another approach the polypeptide is derivatized with multiple polyethylene glycol chains, by covalent attachment of the chains to reactive sites, typically amines, on the polypeptide. The derivatized polypeptide, when administered parenterally, appears to reduce or eliminate the immune response that ordinarily is directed against the free polypeptide. After an initial treatment with the derivatized polypeptide, the immune system is less responsive to the polypeptide in free form, allowing the polypeptide to be administered in free form with reduced immune-response complications (Lee, 1977).

This approach suffers from a potential drawback. Attachment of a large number of polymers to a polypeptide surface may destroy important immunogenic regions, which would then be able to provoke a normal immune response when administered in free form.

4. Summary of the Invention

This invention relates to an improvement in a method for treating a subject by parenteral admininistration of an immunogenic polypeptide. The improvement includes pretreating a subject by parenteral administration of a polymer-polypeptide composition, composed of the polypeptide and polyethylene glycol (PEG) chains which surround, but are not covalently attached to, the polypeptide.

In one embodiment, the polypeptide is attached to a particle support surface and is surrounded by a layer of PEG chains attached to the particle surface. The PEG chains have a molecular weight between about 1,000-10,000 daltons, and the particle to which PEG chains and polypeptides are attached is a liposome.

In various embodiments of polymer-polypeptide compositions of the invention, the polypeptide is: (a) a xenogeneic

antibody, or a xenogeneic antibody F_{ab} fragment, attached to the liposome surface by high-affinity, specific antibody binding to an antigen substrate covalently attached to the liposome surface, (b) an allergen attached to the liposome surface by biotin/avidin high-affinity coupling, or (c) an enzyme attached to the liposome surface by high affinity, irreversible binding to an irreversible enzyme inhibitor.

In another aspect, the invention includes a composition of the type described above.

These and other objects and features of the invention will become more fully apparent when the following detailed description of the invention is read in conjunction with the accompanying drawings.

Brief Description of the Drawings

Figs. 1A-1E are representations of liposome polymer-polypeptide compositions, in which (a) a polypeptide is directly attached to a liposome surface (Fig. 1A), (b) a polypeptide is attached to a liposome surface by a short polyethylene glycol (PEG) spacer arm (Fig. 1B), (c) a polypeptide is bound to a liposome surface by high-affinity binding to an antigen attached to the liposome surface (Fig. 1C), (d) a polypeptide is bound to a liposome surface by high-affinity binding to an enzyme inhibitor attached to the liposome surface (Fig. 1D), and (e) a polypeptide is bound by biotin/avidin coupling to a liposome having surface-bound biotin;

Fig. 2 illustrates a step in the formation of a phosphatidylethanolamine (PE) whose polar head group is derivatized with a maleimide group;

Fig. 3 illustrates a step in the formation of a PE whose polar head group is derivatized with a bromoacetamide group;

Fig. 4 shows steps in forming of a PE derivatized by a short PEG spacer chain having a reactive maleimide group at its free

end;

Fig. 5 shows steps in forming of a PE derivatized by a short PEG spacer chain having an aldehyde group at its free end;

Fig. 6 shows steps in forming a PE derivatized by a PEG spacer chain having a hydrazine group at its free end;

Fig. 7 shows a reaction for covalent attachment of a polypeptide, via a sulhydryl group, to a maleimide group of a derivatized PE;

Fig. 8 shows a reaction for covalent coupling of a polypeptide to a derivatized PE having aldehyde groups via reductive amination;

Figs. 9A-9C illustrate the preparation of a biotinylated PE (Fig. 9A) for use in preparing liposomes with surface-bound biotin, the preparation of a biotinylated IgG (Fig. 9B), and binding of the biotinylated IgG molecule to a liposome surface containing biotinylated PE via high affinity interactions between surface-bound biotin, avidin, and biotin attached to IgG (Fig. 9C); and

Fig. 10 shows a plot of a time course of gallium-67 labelled liposomes composed of hydrazide PEG-DSPE, partially hydrogenated egg phosphatidylcholine (PHEPC), and cholesterol (PEG-HZ fluid liposomes), or hydrazide PEG-DSPE, hydrogenated serum phosphatidylcholine (HSPC), and cholesterol (PEG-HZ rigid liposomes) in the bloodstream.

Detailed Description of the Invention

I. Definitions

"Vesicle-forming lipid" refers to any lipid capable of forming part of a stable micelle or liposome composition and typically including two hydrophobic acyl hydrocarbon chains or a steroid group and a polar group which contains a reactive chemical group, such as an amine, acid, ester, aldehyde or alcohol.

"Allergen" refers to a polypeptide capable of eliciting an immune response, typically involving immunoglobulin E (IgE), when a subject is exposed parenterally to the allergen.

"Antigen" refers to a polypeptide capable of eliciting an response, typically involving an IgG or IgM immune response, when a subject is exposed parenterally to the antigen.

"Immunogen" refers to either an allergen or an antigen.

For purposes of this invention the term "polypeptide" refers to both to peptides, e.g., having less than 50 amino acid residues, and to larger polypeptides, e.g., having up to 200 amino acid residues or even larger.

"Substrate" refers to a compound attached to a liposome surface capable of binding, covalently or nocovalently, a polypeptide to a liposome surface.

"High-affinity binding" refers to substrate binding to a polypeptide with an affinity constant greater than 10^6 M^{-1}, and preferably greater than 10^9 M^{-1}. Examples of such high-affinity binding between substrate and polypeptide, include binding of biotin to avidin, and binding of an antigen to its antibody.

Additionally, "irreversible binding" refers to high-affinity substrate binding by a polypeptide, where the substrate-polypeptide complex is stable and is characterized by a lifetime of at least 48 hours due to slow dissociation of substrate from the polypeptide. Irreversible binding can occur by covalent attachment of a substrate to an enzyme, or by high affinity noncovalent binding of a substrate to an enzyme. Examples of such irreversible binding between substrate and enzyme, include covalent complex formation between organic fluorophosphates and acetylcholinesterase, and vanadate binding to nucleotide-hydrolyzing enzymes.

II. Polymer-Polypeptide Compositions

Figures 1A-1E illustrate various embodiments of a liposomal polymer-polypeptide composition for use in the invention. The composition will be described in its general features with respect to to Fig. 1A. The figure shows a portion of the outer bilayer 10 of a liposome 12 having an outer surface 14. Typically, the liposome will include additional bilayers which are within the outer layer shown. The outer bilayer itself is composed of confronting lipid layers 10a and 10b which are the interior and exterior lipid layers, respectively, of the billayer, each layer being composed of vesicle-forming lipids, such as phospholipids and cholesterol. Methods for forming liposomes suitable for use in the composition are described below. More generally, the liposome is representative of a particle support forming part of the composition.

The composition includes molecules of a polypeptide, such as polypeptide 16 which is attached to the outer liposome surface by covalent coupling to one of the lipids, such as indicated at 18, forming the outer layer of the outer bilayer. Polypeptides suitable for use in the invention, and methods of their preparation are described below. In the embodiment shown, the polypeptide is attached by direct covalent coupling to the polar head group of a lipid, such as lipid 18, in the outer layer of the liposome's outer bilayer. In the embodiments discussed below, other methods of attaching polypeptides to the outer surfaces of liposomes are described.

According to an important feature of the invention, the polypeptide molecules attached to the liposome outer surface are surrounded by, but not covalently linked to, hydrophilic polymer chains, such as chains 20, which are also carried on the liposome's outer surface. The polymer chains are preferably polyethylene glycol (PEG) chains having molecular weights between about 1,000 and 10,000 daltons, correspondig to polymer chain lengths of about 22 to 220 ethylene oxide units.

The polymer chains are covalently attached to the polar head groups of vesicle-forming lipids in the outer layer of the liposome's outer bilayer. Methods of forming PEG-derivatized lipids will be described below. As seen from the figure, the polymer chains form a layer 22 whose thickness is sufficient to cover at least a portion of the polypeptide. Typically, the PEG chains are selected in length so that the layer formed by the polymers will cover all or most of the polypeptide attached to the liposome surface. For example, a polymer layer composed of PEG chains having a molecular weight of about 2,000 daltons has an apparent layer thickness of about 50 angstroms (Needham). This polymer layer would therefore be effective in covering a globular polypeptide having rough dimensions of less than about 50 A diameter.

In one particular embodiment, illustrated in Figure 1B, the polypeptide in the composition is attached to the liposome outer surface by a short polymer chain. The figure shows a liposome bilayer portion 24 with a layer 26 of PEG chains, as above. The polypeptide, such as polypeptide 28, is attached to the liposome outer surface by a spacer chain, such as chain 30. The spacer chain is preferably a short hydrophilic chain, such as a 100-500 dalton PEG chain, which is itself coupled to the polar head group of a lipid, such as lipid 32, in the outer layer 34 of the liposome bilayer. The short spacer chain contains a derivatizable group 36 at its free end.

The present embodiment allows the polypeptide in the polymer layer to be positioned at a selected depth in the layer, as shown, to increase or decrease the extent to which the polypeptide is buried in the polymer layer. For example, in a polymer layer having a thickness of about 50 A, a small polypeptide, e.g., one having a globular size of less than 20 A, may be advantageously placed adjacent the outer surface of the layer by a 100-500 dalton PEG spacer chain, as shown. As in the

general composition described with respect to Fig. 1A, the polpeptide is surrounded by, but not covalently linked to, the PEG chains which form the polymer layer on the liposomes.

In one particular embodiment, illustrated in Figure 1C, the polypeptide in the composition is an antibody or antibody fragment attached to the liposome outer surface by specific, high-affinity binding to an antigen, or antigenic substrate, carried on the liposome outer surface. The figure shows a liposome bilayer portion 38 with a layer 40 of PEG chains, as above. A polypeptide, such as polypeptide 42, is attached to the liposome outer surface by high-affinity binding to an antigen, such as antigen 44, covalently attached to to the polar head group of a lipid, such as lipid 46, in the outer layer of the liposome bilayer.

As in the general composition described with respect to Fig. 1A, the polpeptide is surrounded by, but not covalently linked to, the PEG chains which form the polymer layer on the liposome. An important feature of this embodiment of the invention is that polypeptides exhibit high affinity for the antigenic substrates attached to the liposome surface. These sites on the liposome surface which exhibit a high affinity for the polypeptide will likely facilitate polypeptide binding to the liposome surface.

In another embodiment, illustrated in Figure 1D, the polypeptide in the composition is an enzyme which is attached to the liposome outer surface by specific, high-affinity binding to an enzyme inhibitor carried on an outer liposome surface. The figure shows a liposome bilayer portion 48 with a layer 50 of PEG chains, as above. In this embodiment, the liposome, contains, on or close to its surface 52, numerous enzyme inhibitor molecules, such as enzyme inhibitor 54.

A polypeptide, such as polypeptide 56, binds with high affinity to the enzyme inhibitor molecule, and once bound, the polypeptide will remain substantially bound to the liposome

surface, with a very slow dissociation rate if any. As in the composition described with respect to Fig. 1C, the enzyme is surrounded by, but not covalently linked to, the PEG chains which form the polymer layer on the liposome surface. An important feature of this composition is that enzymes having a high affinity for the enzyme inhibitors may be attached to the liposome surface with a polymer layer by covalent or noncovalent inhibitor substrate binding.

In a final embodiment, illustrated in Figure 1E, the polypeptide in the composition is a biotinylated polypeptide attached to the liposome outer surface by specific, high-affinity binding to avidin carried on the liposome outer surface. The avidin is bound noncovalently to the liposome outer surface by high-affinity interactions with biotin which has been used to derivatize lipid head groups on the liposome surface.

The figure shows a liposome bilayer portion 58 with a layer 60 of PEG chains, as above. The liposome outer surface contains a number of lipid polar head groups, such as lipid polar head group 62, which have been derivatized by biotin. To a biotin molecule, such as biotin molecule 64, is bound an avidin molecule, such as avidin molecule 66. Each avidin molecule contains four high-affinity biotin binding sites, such as biotin binding site 68. To one of these sites is attached the liposome bound biotin as previously indicated. To one or more of the free-remaining sites can be bound a biotinylated polypeptide, such as biotinylated polypeptide 70, which is derivatized by a biotin molecule, such as biotin molecule 72.

As in all the above-described compositions, the polypeptide is surrounded by, but not covalently linked to, the PEG chains which form the polymer layer on the liposome. An important feature of this embodiment of the invention is high affinity of avidin for biotin which makes possible the attachment of

polypeptides to a liposome surface with a polymer layer.

Although the composition of the invention has been described above with respect to a liposomal composition, i.e., one having a liposomal particle support, it will be recognized that a variety of other particles supports may be used. In general, the particle supports should be biodegradable, have surface chemical groups through which polymer chains of polypeptide molecules can be attached to the surface, and preferably have sizes in a selected size range between about 50-500 nm. Exemplary particle supports of this type include sized polylactic acid particles, or microcapsules formed of poly amino acids.

III. Preparation of the Polymer-Polypeptide Composition

This section will describe the preparation of a liposomal composition of the type described in Section II. It will be recognized by one skilled in the art how the methods may be adapted to preparing particle compositions in which the particle support is other than liposomal.

A. Lipid Components

The liposome particle support is composed of three general types of vesicle-forming lipid components. The first includes vesicle-forming lipids which will form the bulk of the vesicle structure in the liposome.

Generally, these vesicle-forming lipids include any amphipathic lipids having hydrophobic and polar head group moieties, and which (a) can form spontaneously into bilayer vesicles in water, as exemplified by phospholipids, or (b) are stably incorporated into lipid bilayers, with its hydrophobic moiety in contact with the interior, hydrophobic region of the bilayer membrane, and its polar head group moiety oriented toward the exterior, polar surface of the membrane.

The vesicle-forming lipids of this type are preferably ones having two hydrocarbon chains, typically acyl chains, and a polar head group. Included in this class are the phospholipids, such as phosphatidylcholine (PC), PE, phosphatidylglycerol (PG), phosphatidic acid (PA), phosphatidylinositol (PI), and sphingomyelin (SM), where the two hydrocarbon chains are typically between about 14-22 carbon atoms in length, and have varying degrees of unsaturation. The above-described lipids and phospholipids whose acyl chains have a variety of degrees of saturation can be obtained commercially, or prepared according to published methods. Other lipids that can be included in the invention are glycolipids and sterols, such as cholesterol.

The second general component includes a vesicle-forming lipid which is derivatized with a polymer chain which will form the polymer layer in the composition. The vesicle-forming lipids which can be used as the second general vesicle-forming lipid component are any of those described for the first general vesicle-forming lipid component. Vesicle forming lipids with diacyl chains, such as phospholipids, are preferred. One exemplary phospholipid is phosphatidylethanolamine (PE), which provides a reactive amino group which is convenient for coupling to the activated polymers. An exemplary PE is distearyl PE (DSPE).

The preferred hydrophilic polymer in the derivatized lipid, is polyethyleneglycol (PEG), preferably a PEG chain having a molecular weight between 1,000-10,000 daltons, more preferably between 2,000 and 5,000 daltons. Other hydrophilic polymers which may be suitable include polyvinylpyrrolidone, polyoxazoline, polymethacrylate, and polydimethylacrylamide, polylactic acid, polyglycolic acid, and derivatized celluloses, such as hydroxymethylcellulose or hydroxyethylcellulose. Additionally, block copolymers or random copolymers of these polymers, particularly including PEG segments, may be suitable.

Methods for preparing lipids derivatized with hydrophilic polymers, such as PEG, are well knowm e.g., as described in co-owned U.S. Patent No. 5,013,556.

The third general vesicle-forming lipid component is a lipid anchor which serves to attach the polypeptide in the composition to the liposome surface. The lipid anchor has a hydrophobic moiety which serves to anchor the lipid in the outer layer of the liposome bilayer surface, and a head group which may be (i) a polar head group which can be activated for covalent coupling to the polypeptide, (ii) a hydrophilic polymer spacer arm which carries an activatable chemical group at its free end, or (iii) a substrate by which the polypeptide can be attached to the lipid component via high-affinity binding, where the substrate may be attached directly to the lipid of through a spacer arm.

Lipid polar head groups which can be activated for coupling of a polypeptide include amine, acid, and hydroxyl groups. One preferred lipid is phosphatidylinositol (PI) which has hydroxyl groups which can be oxidized to reactive aldehyde groups.

Lipid anchors having a spacer arm with a reactive free end are preferably lipids which are derivatized with PEG, as above, but where the PEG is substantially shorter than the PEG forming the polymer layer. For example, in a liposome composition containing a layer formed by PEG polymers of 2,000-5,000 daltons, the spacer arm is generally of 100-1,500 daltons, preferably 600-1,000 daltons.

Lipid anchors in which the head group is a substrate can be prepared by a variety of well-known methods for attaching a substrate, such as biotin, short peptides, or non-peptide enzyme substrates to the polar head groups of lipids. For example, the coupling methods discussed below with respect to coupling polypeptides to activated lipid groups, after liposome formation, are suitable for attaching peptide substrates to free lipids. Other lipid anchors, such as biotinylated PE, are

commercially available. Typically, the substrates are relatively small, e.g., less than about 5,000 daltons, to allow their incorporation into multilamellar liposomes with a minimum of disruption of the lipid bilayer structures. The substrate is preferably one capable of binding irreversibly to the polypeptide, to ensure that the polypeptide remains bound to the liposomes over its lifetime in the bloodstream.

B. Liposome Preparation

The liposomes may be prepared by a variety of techniques, such as those detailed in Szoka et al, 1980. Multilamellar vesicles (MLVs) can be formed by simple lipid-film hydration techniques. In this procedure, a mixture of liposome-forming lipids of the type detailed above dissolved in a suitable organic solvent is evaporated in a vessel to form a thin film, which is then covered by an aqueous medium. The lipid film hydrates to form MLVs, typically with sizes between about 0.1 to 10 microns.

The lipids components used in forming the liposomes are preferably present in a molar ratio of about 70-90 percent vesicle forming lipids, 1-25 percent polymer derivatized lipid, and 0.1-5 percent lipid anchor. One exemplary formulation includes 50-70 mole percent underivatized PE, 20-40 mole percent cholesterol, 0.1-1 mole percent of a PE-PEG (150) spacer polymer with a chemically reactive group at its free end for polypeptide or substrate coupling, 5-10 mole percent PE derivatized by PEG 3500 polymer chains, and 1 mole percent α-tocopherol.

The liposomes are preferably prepared to have substantially homogeneous sizes in a selected size range, typically between about 0.03 to 0.5 microns. One effective sizing method for REVs and MLVs involves extruding an aqueous suspension of the liposomes through a series of polycarbonate membranes having a selected uniform pore size in the range of 0.03 to 0.2 micron,

typically 0.05, 0.08, 0.1, or 0.2 microns. The pore size of the membrane corresponds roughly to the largest sizes of liposomes produced by extrusion through that membrane, particularly where the preparation is extruded two or more times through the same membrane. Homogenization methods are also useful for downsizing liposomes to sizes of 100nm or less (Martin).

C. Polypeptide Components

The polypeptide in the composition is an immunogenic polypeptide. This polypeptide can be an antibody, or F_{ab} antibody fragment. These antibodies may be obtained from nonhuman sources, or may be chimeric polypeptides containing peptide regions derived from both human and nonhuman sources. The antibodies may be conjugated with a toxin, a radionuclide, or a chemotherapeutic drug.

Exemplary antibodies include murine monoclonal antibodies to (i) tumor-specific antigens used for therapy of cancer of the colon, ovary, or breast, (ii) CD3 for treatment of renal allograft rejection, or (iii) IL-2 for treatment of autoimmune diseases (Sehon, 1992).

In another general embodiment, the polypeptide is a therapeutic enzyme, such as streptokinase, tissue plasminogen activator (TPA), or arginase. These polypeptides, either because of their species origin, or because of their method of preparation, have the potential, when administered in free form, to induce an immune response in a human subject.

In still another general embodiment, the polypeptide is an allergen, such as an isolated protein or protein fragment from a known allergenic source. Example include ovalbumin, or a mixture of allergenic peptides, such as nondialysable constituents of an aqueous extract of ragweed pollen.

D. Attachment of Polypeptides to Liposome Surfaces

Polypeptide compositions of this invention can be attached to liposome surfaces by covalent attachment or by polypeptide binding to a substrate carried on the liposome surfaces.

1. Covalent Attachment Methods

In this approach, the lipid anchor components in the liposomes are reacted with the polypeptide under conditions favoring covalent attachment to the lipid anchor. In one general approach, the polar head group of the lipid anchor is activated by a suitable activating agent, and the activated liposomes are then reacted with the polypeptide. It will be appreciated that both the activation and polypeptide-coupling reactions are carried out in an aqueous environment compatible with liposome integrity.

Fig. 2 illustrates the activation of DSPE (compound I) by maleimido propionate N-hydroxysuccinimide ester (compound II) (MPS, Pierce), to form an activated maleimido-DSPE (compound III). The maleimido group at the polar head group is reactive towards thiol-containing polypeptides, to attach the polypeptide to the liposome surface via a thioether linkage.

Fig. 3 illustrates the activation of DSPE (compound I) by bromoacetyl N-hydroxysuccinimide ester (compound IV) to form the bromo acetamide of DSPE (compound V). The bromoacetamide group at the polar head group is also reactive towards thiol-containing polypeptides, to attach the polypeptide to the liposome surface via a thioether linkage.

Fig. 4 shows the synthesis of a DSPE derivatized with a PEG chain and having an activated chemical group at the chain's free end. Initially, PEG bis (amine) (compound VI), protected at one end by 2-nitrobenzene sulfonyl chloride (compound VII), is reacted with carbonyl diimidazole in triethylamine (TEA) to form the imidazole carbamate of the mono 2-nitrobenzenesulfonamide (compound VIII). The compound is reacted with DSPE in TEA to

form the derivatized PE lipid protected at one end with 2-nitrobenzyl sulfonyl chloride. The protective group is removed by treatment with acid to give the DSPE-PEG (compound IX) having a terminal amine on the PEG chain. Reaction with maleic acid anhydride gives the corresponding maleamic (compound X), which on reaction with acetic anhydride gives the PE-PEG-maleimide (compound XI). Details of the reactions are given in Example 1.

The compound is reactive with sulhydryl groups, for coupling polypeptides through a thioether linkage to generate compound XXI, as illustrated in Fig. 7.

Another reaction method for coupling a protected polyalkylether to a lipid amine is shown in Fig. 5. In this reaction scheme, PEG (compound XII) is initially protected at one of its terminal OH ends by a trimethylsilane group, as shown at the top in Fig. 5. The protected PEG (compound XIII) is reacted with the anhydride of trifluoromethyl sulfonate to activate the free PEG end with trifluoromethyl sulfonate (compound XIV). Reaction of the activated compound with a lipid amine, such as PE, in the presence of triethylamine, gives the desired derivatized PE product.

The trimethylsilyl protective group can be released by acid treatment, yielding the desired PE-PEG (compound XV) with a free terminal OH. Reaction of compound XV with acetic anhydride in DMSO converts the terminal OH to an aldehyde group (compound XVI) which can be coupled to a peptide via reductive amination, as illustrated in Fig. 8, to generate compound XXIII. Reaction details are given in Example 2.

The aldehyde-based coupling reaction just described may also be employed in a liposome composition containing PI, PG, or other lipid anchor component with an oxidizable head group. In this approach, the preformed liposomes are oxidized, e.g., by reaction with periodate. The aldehyde groups formed by the oxidation may then be used for protein coupling, as illustrated

in Fig. 8.

The procedure illustrated in Figure 6 describes the preparation of DSPE-PEG-hydrazide. First PEG is reacted with ethyl isocyanatoacetate in the presence of triethylamine to generate mono and dicarboxylated species of PEG. The monocarboxylated species is purified by ion-exchange chromatography on DEAE-Sephadex (compound XVII). Compound XVII is reacted with tert-butyl carbazate to generate the hydroxy Boc-hydrazide derivative of PEG (compound XVIII). The free hydroxyl group is activated by reaction with disuccinimidyl carbonate to activate the terminal hydroxyl group (compound XIX) prior to reaction with DSPE to generate product (compound XX). Hydrazide groups, obtained by deprotection of compound XX (Boc group removal) are reactive towards aldehydes, which can be generated on numerous biologically relevant compounds. Hydrazides can also be acylated by active esters or carbodiimide-activated carboxyl groups. Acyl azide groups reactive as acylating species can be easily obtained from hydrazides thus allowing conjugation of amino containing ligands.

Similar reaction methods can be used for coupling polypeptides to the free ends of PEG spacer arms carried on the liposome surfaces. Experiments carried out in support of the invention indicate that more efficient coupling of polypeptides to the liposomes may occur as the length of the spacer arm is increased.

2. Noncovalent Attachment Methods

One exemplary method for attaching polypeptides noncovalently to liposomes is illustrated in Figs. 9A-9C. Fig. 9A shows the preparation of a biotinylated DSPE lipid anchor (compound XXV) which is incorporated into liposomes as described above. Fig. 9B shows a similar reaction for biotinylating a

polypeptide, in this case, an IgG to generate compound XXVI. Coupling is achieved by first binding avidin to the liposomes, then incubating the avidin-coated liposomes with the polypeptide. Experiments conducted in support of the present invention indicate that polypeptides are efficiently attached to PEG-coated liposomes by this method. Details are given in Example 4.

Similarly, where the lipid anchor in the liposomes is a substrate, the liposomes are incubated with the polypeptide under conditions effective to allow polypeptide binding to the substrate.

IV. Bloodstream and Tissue Retention of Liposomes Containing End-functionalized PEG-DSPE

In vivo studies were undertaken to determine the bloodstream and tissue retention of liposomes containing end-functionalized PEG-DSPE. End-functionalized PEG-DSPE contains a chemically active group which can be used for attaching a variety of compounds to liposomes. From these studies it has been determined that end-functionalization does not affect the extended lifetime in the bloodstream of liposomes containing PEG-DSPE, monomethoxy PEG-DSPE, or other similarly modified vesicle-forming lipids.

In experiments performed in support of the present invention, liposomes containing PEG-DSPE end-functionalized by hydrazide were prepared. The hydrazide group at the end of a PEG chain can be used for the introduction of other functional groups, or can be used in numerous types of conjugation schemes (Inman). Particularly useful is hydrazide's reactivity toward various glycoproteins, such as immunoglobulins (Wilchek), for attaching these molecules to liposomes.

Gallium 67-labelled, hydrazide end-functionalized PEG liposomes were injected in rats by tail vein injection at about

10-20 micromolar phospholipid/kg body weight. Blood sample were obtained by retroobital bleeding at defined times. The percent of gallium labelled liposomes remaining in the bloodstream was determined at 0, 15 minutes, 1, 3, 5, and 24 hours and is presented in Table 1. The percent injected gallium 67-labelled liposome dose remaining in the blood stream at different times is illustrated in a half log plot versus time in Fig. 10.

After 24 hours the animals were sacrificed and tissues removed for label quantitation. The percent of the injected dose found in selected tissues at 24 hours is presented in Table 1.

The blood and tissue retention of Ga-labelled, hydrazide end-functionalized liposomes having two different lipid compositions were also compared in Table 1. A fluid liposome composition was prepared from partially hydrogenated egg phosphatidylcholine (HPEPC). A typical liposome composition contains the hydrazide PEG-DSPE lipid, partially hydrogenated egg PC (PHEPC), and cholesterol in a lipid:lipid:lipid mole ratio of about 0.15:1.85:1. A rigid liposome composition was prepared by substituting hydrogenated serum phosphatidylcholine (HSPC) for PHEPC at the same mole ratio.

As is indicated in Table 1, the fluidity of the liposome composition does not affect the blood retention time of the liposomes. However, the fluidity of the liposome composition does appear to affect the tissue distribution of the end-functionalized liposome. For example, rigid liposomes are preferentially retained by live, spleen and bone tissue. Fluid liposomes are preferentially retained by the kidneys, heart, skin and muscle tissue.

Table 1

% Injected 67 GA Dose Detected at Specified Timepoints		
Blood	Peg-HZ Rigid	PEG-Hz Fluid
0	101.1 ± 12.0	100.2 ± 5.4
15 min.	89.6 ± 11.2	81.6 ± 2.5
1 hr.	84 ± 11.1	81.7 ± 7.4
3 hr.	76 ± 10.5	75.3 ± 5.1
5 hr.	71.7 ± 10.7	66.3 ± 3.8
24 hr.	33.4 ± 6.8	34.3 ± 0.68
Tissues at 24 hr.	Peg-HZ Rigid	Peg-Hz Fluid
liver	12.1 ± 1.2	8.8 ± 0.81
spleen	5.1 ± 0.47	4.7 ± 0.64
kidneys	1.4 ± 0.22	1.7 ± 0.25
heart	0.36 ± 0.037	0.77 ± 0.21
lungs	.62 ± 0.23	0.58 ± 0.03
skin	.086 ± 0.03	0.16 ± 0.08
muscle	.08 ± 0.03	0.29 ± 0.02
bone	.28 ± 0.09	0.04 ± 0.01

V. Method of Treatment

In one aspect, the invention includes an improved method for treating a subject by parenteral administration of an immunogenic polypeptide. The improvement includes pretreating the subject by parenteral administration of a polymer-

polypeptide composition of the type described above, i.e., a composition containing the polypeptide and polyethylene glycol (PEG) chains which surround, but are not covelently attached to, said polypeptide.

Typically, the polymer-polypeptide composition is administered intravenously several days before treatment with the peptide in free form. This pretreatment is designed to desensitize the subject to the peptide, i.e., suppress the subject's immune response to the free peptide.

The composition is preferably injected in an amount corresponding to between about 0.1 to 2 mg polypeptide/kg body weight. The composition may be administered at periodic intervals during the pretreatment period. After the pretreatment period, the peptide in free form can be administered for periods of up to 20 and 40 days, with reduced immune response to the free polypeptide.

In order to maintain the subject in a immune-tolerant condition, the polymer-polypeptide composition can be administered at intervals throughout the treatment.

The following examples describe the preparation of exemplary polymer-polypeptide compositions. The examples are intended to illustrate, but in no way limit, the invention as claimed.

Example 1

Preparation of DSPE-PEG-Maleimide

A. Preparation of the Mono 2-nitrobenzenesulfonamide of PEG bis(amine) (compound VII).

A mixture of 1.7 g (0.5 mmole) of commercially available polyethylene glycol bis(amine) and 104 mg (0.55 mmole) of 2-nitrobenzene sulfonyl chloride were added to a round bottomed flask. The minimum amount of dioxane to effect solution (about 15 ml) and 280 microliters of triethylamine (2 mmole) were

added. The reaction flask was stoppered and let to stand at room temperature for 4 days.

Thin layer chromatography (TLC) on SiO2 coated plates using a solvent mixture of the following composition CHCl3/ CH3OH/ H2O/ NH4OH; 130/ 70/ 8/ 0.5; v/v/v/v showed fluorescence quenching spots at Rf= 0.87 to 0.95 and Rf=0.68-0.75. The 2-nitro benzene sulfonyl chloride was a more compact spot at Rf=0.85. The UV absorbing material at Rf= 0.87-0.95 probably represented the bis-2-nitro-benzenesulfenamide. The material at Rf=0.68-0.75 probably represented the desired mono-2-nitrobenzenesulfonaimde of the starting diamine.

The solvent was evaporated under vacuum to obtain 2.135 g of a yellow syrup. It was dissolved in 5 ml chloroform and placed at the top of a 21 mm x 270 mm column of SiO2 wetted with chloroform. The product was purified by passing through the column, in sequence:

100 ml	100% chloroform	0% (1% conc. NH_4OH in MeOH)
200 ml	90% "	10% "
100 ml	80% "	20% "
100 ml	70% "	30% "

Fifty ml aliquots were collected separately and assayed by TLC as described above. Most of the yellow, ninhydrin positive-reacting material was eluted in the 20% (1% conc. NH_4OH in MeOH) fraction. The fractions were dried and resulted in 397 mg of a bright yellow solid. The yield of the pure sample was about 20%

B. Preparation of the Imidazole Carbamate of the Mono 2-nitrobenzenesulfonamide of PEG bis(amine)(compound VIII).

550 mg (0.15 mmole) of the 2-nitrobenzenesulfonamide of PEG bis(amine) were dissolved in anhydrous benzene. To this was added 49 mg of carbonyl diimidazole (0.3 mmole) and 28 microliters (0.20 mmole) of triethylamine. The air in the reaction vessel was displaced with nitrogen, the flask stoppered and heated in an 80 degree oil bath

for 4 hours. TLC on silicate-coated plates using the same solvent system as described above, showed that all the starting sulfonamide (Rf=0.72) had been consumed, and had been replaced by an iodine absorbing material at Rf=0.92.

The solvent was removed under vacuum. The residue was dissolved in about 2.5 ml chloroform and transferred to the top of a 21 x280 mm cloumn of silicate which was wetted with chloroform. The following solvents were passed through the column, in sequence:

100 ml	100%	chloroform	0% (1% conc. NH_4OH in CH_3OH)
100 ml	90%	"	10% "
200 ml	80%	"	20% "

50 ml fractions were collected and assayed by TLC, the desired product was found predominantly in the 20% (1% conc. NH_4OH in CH3OH fraction). When the faction was evaporated to dryness, the sample afforded 475 mg of a lemon-yellow solid. This was dissolved in 4.75 ml benzene.

C. Preparation of the DSPE carbamide of the 2-nitrobenzene sulfonamide of PEG bis(amine).

To the 450 mg (0.125 mmole) of 2-nitrobenzenesulfonamide of the imidazole carbamide of the PEG bis(amine) dissolved in 4.5 ml benzene was added 93 mg DSPE (0.125 mmole) and 70 microliters (0.50 mmole) of triethylamine. The air was displaced with nitrogen, the flask stoppered and heated in an oil bath at 80 degrees for 6 hours. The flask was cooled to room temperature. DSPE migrates in the above described TLC system with an Rf of 0.54. TLC indicated that all the DSPE had been consumed. The solvent was evaporated under vacuum. The residue was dissolved in 2.5 ml chloroform and placed at the top of a 21 x 260 mm column of silicate wetted with chloroform. The sample was purified by passing through the column in sequence:

100 ml 100% chloroform 0% (1% conc. NH_4OH in MeOH)

200 ml	90%	"	10%	"
100 ml	80%	"	20%	"
100 ml	70%	"	30%	"

The desired product eluted at 20% (1% conc. NH_4OH in MeOH), was evaporated and afforded 358 mg of a bright yellow solid with an Rf= 0.95. Fractions containing imidazole were not used and the final yield of the product (0.0837 mmoles) was 65%.

D. Preparation of the DSPE Carbamide of PEG bis(amine) (compound IX).

About 358 mg of nitrobenzenesulfenamide of the DSPE carbamate of polyethyleneglycol bis (amine) were dissolved in 10 ml ethanol. To the solution weere added 2.4 ml water and 1.2 ml acetic acid. The mixture was allowed to stand at room temperature for 18 hours. TLC analysis showed only partial deprotection. Another 2.3 ml water, and another 1.2 ml acetic acid were added and the reaction was left standing overnight. On silicate coated plates, using a similar solvent system as described above as the developer, flourescence quenching material appeared at Rf=0.86 and Rf=0.74. The desired ninhydrin reactive, phosphate containing material migrated with an Rf value of 0.637. This spot showed no fluorescence quenching.

The solvent was removed under vacuum. The residue was redissolveed in 15 ml chloroform and extracted with 15 ml 5% sodium carbonate. The mixture was centrifuged to effect separation, and the sodium carbonate phase was reextracted 2x with 15 ml chloroform. The combined chloroform extracts were evaporated under reduced pressure to obtain 386 mg of wax. TLC indicated that the wax was largely a ninhydrin positive, phosphate containing lipid of Rf=0.72.

The wax was dissolved in 2.5 ml chloroform and placed on a

silicate column which had been wetted with chloroform. The following solvents were passed through the column in sequence:

100 ml of	100% chloroform	0% (1% CONC. NH_4OH in methanol)
100 ml	90%	10%
100 ml	80%	20%
100 ml	70%	30%
100 ml	50%	50%
100 ml	0%	100%

The samples were assayed by TLC. The desired product was found in the fractions with 30% and 50% (1% conc. NH_4OH in methanol). These samples were combined and evaporated to dryness under vacuum to afford 91 mg (22 micromoles) of a viscous syrup, which corresponded to about 100% conversion.

E. Preparation of the Maleic Acid Derivative of the DSPE Carbamide of PEG bis(amine) (compound X).

To 18 micromoles of the DSPE carbamide of PEG bis (amine) described above, dissolved in 1.8 ml chloroform, was added 3.5 mg (36 micromoles) maleic anhydride and 5 microliters (36 micromoles) triethylamine. The stoppered flask was allowed to stand at room temperature for 24 hours. The solvent was evaporated. TLC on silicate plates indicated that all the starting material, had been replaced by a ninhydrin negative, phosphate containing material of Rf= 0.79-1.00.

F. Preparation of the Maleimide of the DSPE carbamide of PEG bis (amine) (compound XI).

The syrup was dissolved in 2 mls acetic anhydride saturated with anhydrous sodium acetate. The solution was heated in a 50 degree oil bath for two hours. 10 ml ethanol were added and evaporated under vacuum. This step was repeated twice to remove excess acetic anhydride and acetic acid. Took up the residue in 1 ml chloroform, and passed the sample through a column with the following solvents in sequence:

100 ml	100% chloroform	0% (1% conc. NH_4OH in MeOH)
200 ml	90% "	10% "
100 ml	80% "	20% "
100 ml	70% "	30% "

50 ml samples were collected, and the main product was found in the fractions eluted with 10% of 1% conc. NH_4OH in MeOH. The fractions were combined and then evaporated to dryness under vacuum which afforded 52 mg of a pale, yellow, viscous oil, which by TLC migrated with an Rf of 0.98 and contained phosphate. 12.3 mmoles product were obtained which corresponded to a yield of about 34%.

Example 2

Preparation of the Aldehyde of the Ethylene-Linked PEG-DSPE

A. Preparation of I-trimethylsilyloxy-PEG (compound XII)

15.0 gm (10 mmoles) of PEG) M.Wt. 1500, (Aldrich Chemical) was dissolved in 80 ml benzene. 1.40 ml (11 mmoles) of chlorotrimethyl silane (Aldrich Chemical Co.) and 1.53 ml (1 mmoles) of triethylamine was added. The mixture was stirred at room temperature under an inert atmosphere for 5 hours.

The mixture was filtered by suction to separate crystals of triethylammonium chloride and the crystals were washed with 5 ml benzene. Filtrate and benzene wash liquids were combined. This solution was evaporated to dryness under vacuum to provide 15.83 grams of colorless oil which solidified on standing.

TLC of the product on Si-C_{18} reversed-phase plates using a mixture of 4 volumes of ethanol with 1 volume of water as developer, and iodine vapor visualization, revealed that all the polyglycol 1500 (R_f=0.93) has been consumed, and was replaced by a material of R_f=0.82. An infra-red spectrum revealed absorption peaks characteristic only of polyglycols.

Yield of I-trimethylsilyoxyPEG, M.W. 1500 was nearly

quantitative.

B. Preparation of trifluoromethane sulfonyl ester of trimethylsilyloxy-PEG (compound XIV)

15.74 grams (10 mmol) of the crystalline I-trimethylsilyloxy PEG obtained above was dissolved in 40 ml anhydrous benzene and cooled in a bath of crushed ice. 1.53 ml (11 mmol) triethylamine and 1.85 ml (11 mmol) of trifluoromethanesulfonic anhydride obtained from Aldrich Chemical Co. were added and the mixture was stirred over night under an inert atmosphere until the reaction mixture changed to a brown color.

The solvent was then evaporated under reduced pressure and the residual syrupy paste was diluted to 100.0 ml with methylene chloride. Because of the great reactivity of trifluoromethane sulfonic esters, no further purification of the trifluoromethane sulfonyl ester of I-trimethylsilyloxy PEG was done.

C. Preparation of N-(1-trimethylsilyloxy PEG1500)-PE.

10 ml of the methylene chloride stock solution of the trifluoromethane sulfonyl ester of 1-trimethylsilyloxy PEG was evaporated to dryness under vacuum to obtain about 1.2 grams of residue (approximately 0.7 mmoles). To this residue, 3.72 ml of a chloroform solution containing 372 mg (0.5 mmoles) egg PE was added. To the resulting solution, 139 microliters (1.0 mmole) of triethylamine was added and the solvent was evaporated under vacuum. To the obtained residue, 5 ml dry dimethyl formamide and 70 microliters (0.50 mmoles) triethylamine (VI) was added. Air from the reaction vessel was displaced with nitrogen. The vessel was closed and heated in a sand bath a 110°C for 22 hours. The solvent was evaporated under vacuum to obtain 1.58 grams of brownish colored oil.

A 21 X 260 mm chromatographic absorption column filled with Kieselgel 60 silica 70-230 mesh, was prepared and rinsed

with a solvent composed of 40 volumes of butanone, 25 volumes acetic acid and 5 volumes of water. The crude product was dissolved in 3 ml of the same solvent and transferred to the top of the chromatography column. The chromatogram was developed with the same solvent and sequential 30 ml portions of effluent were assayed each by TLC.

The TLC assay system used silica gel coated glass plates, with solvent combination butanone/acetic acid/water; 40/25/5; v/v/v. Iodine vapor absorption served for visualization. In this solvent system, the N-1-trimethylsilyloxy PEG 1500 PE appeared at R_f=0.78. Unchanged PE appeared at R_f=0.68.

The desired N-1-trimethylsilyloxy PEG 1500 PE was a chief constituent of the 170-300 ml portions of column effluent. When evaporated to dryness under vacuum these portions afforded 111 mg of pale yellow oil of product.

D. Preparation of N-polyethylene glycyl 1500: PE (compound XV).

Once-chromatographed, PE compound was dissolved in 2 ml of tetrahydrofuran. To this, 6 ml acetic acid and 2 ml water was added. The resulting solution was let to stand for 3 days at 23°C. The solvent from the reaction mixture was evaporated under vacuum and dried to constant weight to obtain 75 mg of pale yellow wax. TLC on Si-C18 reversed-phase plates, developed with a mixture of 4 volumes ethanol, 1 volume water, indicated that some free PE and some polyglycol-like material formed during the hydrolysis.

The residue was dissolved in 0.5 ml tetrahydrofuran and diluted with 3 ml of a solution of ethanol water; 80:20; v:v. The mixture was applied to the top of a 10 mm X 250 mm chromatographic absorption column packed with octadecyl bonded phase silica gel and column was developed with ethanol water 80:20% by volume, collecting sequential 20 ml portions of effluent. The effluent was assayed by reversed phase TLC. Fractions

containing only product of Rf=0.08 to 0.15 were combined. This was typically the 20-100 ml portion of effluent. When evaporated to dryness, under vacuum, these portions afforded 33 mg of colorless wax PEG-PE corresponding to a yield of only 3%, based on the starting phosphatidyl ethanolamine.

NMR analysis indicated that the product incorporated both PE residues and PEG residues. The product prepared was used for a preparation of PEG-PE liposomes.

E. Preparation of the Aldehyde of PEG-DSPE (compound XVI).

The free hydroxyl group on PEG derivatized by DSPE can be oxidized to the corresponding aldehyde in the following manner prior to incorporation of the linear polymers into liposomes (Harris). About 2.7 g PEG1500-DSPE (1 mmole), which is prepared as in Example 3, is added to 0.4 g acetic anhydride in 15 ml dimethylsulfoxide with stirring for 30 hours at room temperature. At this point the reaction mixture is neutralized with dilute sodium hydroxide, the solvent is evaporated under reduced pressure.

The residue is dissolved in 10 ml chloroform, washed with two successive 10 ml portions of water, and centrifuged to separate chloroform and water phases. The chloroform phase is evaporated under vacuum to obtain a wax. The wax is re-dissolved in 5 ml chloroform and transferred to the top of a 21 X 270 mm column of silica gel moistened with chloroform. The column is developed by passing 100 ml of solvent through the column. The following solvents were used in sequence:

Volume % Chloroform	Volume % Methanol Containing 2% Conc. Ammonium Hydroxide/methanol
100%	0%
95%	5%
90%	10%
85%	15%
80%	20%
70%	30%
60%	40%
50%	50%
0%	100%

Separated 50 ml fractions of column effluent are saved. The fractions of the column are separated by TLC on Si-C18 reversed-phase plates. TLC plates are developed with 4 volumes of ethanol mixed with 1 volume of water. Visualization is done by exposure to iodine vapor.

Only those fractions containing an iodine-absorbing lipid of R_f about 0.20 were combined and evaporated to dryness under vacuum and dried in high vacuum to constant weight. In this way 94 mg of waxy crystalline solid was obtained of M.W. 2226. The conversion of the terminal alcohol to the aldehyde can be monitored by IR.

Example 3
Preparation of DSPE-PEG-Hydrazide

A. Preparation of ω-Hydroxy Acid Derivative of PEG, α-(Hydroxyethyl)-ω-(carboxymethylaminocarbonyl)oxy-poly(oxyethylene) (Compound XVII).

Polyethylene glycol (Fluka, PEG-2000, 42 g, 42 mequiv OH) is dissolved in toluene (200 ml) and azeotropically dried (Zalipsky, 1987), and treated with ethyl isocyanotoacetate (2.3 ml, 21 mmol) and triethylamine (1.5 ml, 10 mmol). After overnight reaction at 25°C the solution is evaporated to dryness. The residue is dissolved in 0.2 M NaOH (100 ml) and

any trace of toluene is evaporated. The solution is maintained at pH 12 with periodical dropwise additions of 4 M NaOH.

When the solution pH is stabilized at pH 12, the solution is acidified to pH 3.0 and the product is extracted with methylene chloride (100 ml × 2). TLC on silica gel G (isopropyl alcohol/H_2O/conc. ammonia 10:2:1) gives a typical chromatogram of partially carboxylated PEG (Zalipsky, 1990) consisting of unreacted PEG (R_f = 0.67), monocarboxylated derivative (R_f = 0.55) and dicarboxylated derivative of the polymer (R_f = 0.47). This solution is dried over ($MgSO_4$), filtered and evaporated to dryness. The PEG mixture is dissolved in water (50 ml). One-third of this solution (30 ml ≈ 14 g of derivatized PEG) is loaded onto DEAE-Sephadex A-25 (115 ml of gel in borate form). After the underivatized PEG is washed off the column with water (confirmed by negative polymethacrylic acid (PMA) test, (Zalipsky, 1990) gradient of ammonium bicarbonate (2-20 mM at increments of 1-2 mM every 200 ml) was applied, and 50 ml fractions collected. Fractions 1-25 contain only PEG monoacid as determined by PMA and TLC tests. These fractions are pooled together, concentrated to ≈ 70 ml, acidified to pH 2 and extracted with methylene chloride (50 ml × 2). The CH_2Cl_2 solution is dried ($MgSO_4$), concentrated and poured into cold stirring ether. The precipitated product is dried *in vacuo*. Yield: 7 g. Titration of carboxyl groups gives $4.6 \cdot 10^{-4}$ mequiv/g (97% of theoretical value).

B. Preparation of Compound XVIII.

The ω-hydroxy acid derivative of PEG (5 g, 2.38 mmol) and *tert*-butyl carbazate (0.91 g, 6.9 mmol) are dissolved in CH_2Cl_2-ethyl acetate (1:1, 7 ml). The solution is cooled on ice and treated with DCC (0.6 g, 2.9 mmol) predissolved in the same solvent mixture. After 30 minutes the ice bath is

removed and the reaction is allowed to proceed for an additional 3 hours. The reaction mixture is filtered from dicyclohexylurea and evaporated. The product is recovered and purified by two precipitations from ethyl acetate-ether (1:1) and dried *in vacuo* over P_2O_5. Yield: 5.2 g, 98%. TLC of the product gave one spot (R_f = 0.68) instead of the starting material (R_f = 0.55). H-NMR ($CDCl_3$): δ 1.46 (s, t-Bu, 9H); 3.64 (s, PEG, 178H); 3.93 (br. d, J = 4.5, CH_2 of Gly, 2H); 4.24 (t, $C\underline{H}_2$-OCO-Gly, 2H) ppm. ^{13}C-NMR ($CDCl_3$): δ 28.1 (t-Bu); 43.4 (CH_2 of Gly); 61.6 (CH_2OH); 64.3 ($\underline{C}H_2OCONH$); 69.3 ($\underline{C}H_2CH_2OCONH$); 70.5 (PEG); 72.4 ($\underline{C}H_2CH_2OH$); 81.0 ($CMe_3$); 155.1 (C=O of Boc); 156.4 (C=O of Gly urethane; 168.7 (C=O of Gly hydrazide) ppm.

C. Preparation of Compound XIX.

The ω-hydroxy Boc-hydrazide derivative of PEG (5 g, 2.26 mmol) is dissolved in pyridine (1.1 ml), CH_2Cl_2 (5 ml) and CH_3CN (2 ml) and treated with disuccinimidyl carbonate (1.4 g, 5.5 mmol) at 25°C overnight. The solution is filtered and gradually added to cold ethyl ether (100 ml). The precipitated product is dissolved in warm ethyl acetate (45 ml), chilled and mixed with equal volume of ethyl ether. The precipitate is collected by filtration and dried *in vacuo* over P_2O_5. Yield: 4.8 g, 90%. Succinimidyl carbonate groups content $4.15 \cdot 10^{-4}$ mequiv/g (98% of theoretical value) was determined by titration (Zalipsky, 1991). H-NMR ($CDCl_3$): d 1.46 (s, t-Bu, 9H); 2.83 (s, succinimide); 3.64 (s, PEG, 178H); 3.79 (t, $C\underline{H}_2CH_2OCO_2$-Su); 3.93 (br. d, J = 4.5, CH_2 of Gly, 2H); 4.24 (t, $C\underline{H}_2$-OCO-Gly, 2H); 4.46 (t, $C\underline{H}_2OCO_2$-Su) ppm.

D. Preparation of Compound XX. To prepare the DSPE-PEG-Hydrazide a slight excess of succinimidyl carbonate Boc-protected PEG-glycine hydrazide, prepared above, is reacted with DSPE suspended in chloroform in the presence of triethylamine. The lipid derivative is quickly (5-10 minutes)

solubilized in the process of this reaction. The excess of heterobifunctional PEG is removed by dialysis using 300,000 MWCO cellulose ester dialysis membrane from Spectrum. The recovered lipid conjugate was subjected to conventional Boc-deprotection conditions (4M HCl in dioxane) and then further purified by recrystallization.

Example 4

Preparation of Liposomes with Noncovalently Bound IgG

A. Preparation of Biotin-PE (compound XIX)..

Phosphatidylethanolamine (PE) is biotinylated by incubating PE (5 mmole) in dimethylsulfoxide (DMSO) with N-hydroxysuccinimide biotin (5 mmole) at room temperature for four hours. Biotin-PE is utilized in forming liposomes by the reverse phase evaporation method.

Liposomes containing 1 mole percent biotin-PE are incubated in the presence of avidin. Liposome-bound avidin is separated from free avidin on a Sepharose CL-B column (25 x 1 cm) in TES-buffered saline (pH 7.4).

B. Preparation of Biotinylated IgGs (compound XX).

Antibody, 5 mg IgG in 1 ml sodium borate buffer (0.1 M, ph8.8), is incubated with 100 microliters N-hydroxysuccinimide biotin (10 mg/ml in dimethyl sulfoxide) at room temperature for four hours. Biotinylated antibodies are purified by passage through a Sephadex G-25 column (25 x 1 cm) in TES buffered saline.

C. Preparation of Liposome-bound IgGs.

Avidin-coated liposomes are incubated with biotinylated antibodies for 30 minutes. Liposome bound antibody is separated from free antibody on a Sepharose CL-4B column (40 x 1 cm) in TES-buffered saline.

Example 5

Liposome Blood Lifetime Measurements of Hydrazide End -functionalized PEG Liposomes

A. Preparation of Hydrazide End-functionalized Liposomes

Hydrazide PEG-DSPE composed of PEG, end-functionalized with a hydrazide group, and distearyl-PE was prepared as described. The hydrazide PEG-DSPE lipid was combined with partially hydrogenated egg PC (PHEPC) and cholesterol in a lipid:lipid:lipid mole ratio of about 0.15:1.85:1 and the lipid mixture was hydrated. Generally, lipid hydration occured in the presence of desferal mesylate, followed by sizing to 0.1 micron, and removal of non-entrapped desferal by gel filtration with subsequent loading of Ga-oxide into the liposomes. The unencapsulated Ga was removed during passage through a Sephadex G-50 gel exclusion column. Both compositions contained 10 micromoles/ml in 0.15 M NaCl, 5 mM desferal.

A second lipid mixture was prepared in a similar manner but with HSPC instead of PHEPC.

B. Measuring Blood Circulation Time and Tissue Levels.

In vivo studies of liposomes were performed in laboratory rats at 200-300 g each. These studies involved tail vein injection of liposome samples at about 10-20 micromolar phospholipid/kg body weight. Blood sample were obtained by retroobital bleeding at defined times. The animals were sacrificed after 24 hours and tissues removed for label quantitation. The weight and percent of the injected dose in each tissue was determined. The studies were carried out using ^{67}Ga-desferal loaded liposomes and radioactivity was measured using a gamma counter. The percent of the injected dose remaining in the blood at several time points up to 24 hours, and in selected tissues at 24 hours was determined.

1. Plasma Kinetics of Hydrazide-PEG Liposomes.

The liposome composition (0.4 ml) was injected IV in animals. At times 0, 0.25, 1, 3, or 5 and 24 hours after injection, blood samples were removed and assayed for the amount of Ga-desferal remaining in the blood, expressed as a percentage of the amount measured immediately after injection. Hydrazide-PEG liposome have a blood halflife of about 15 hours, and nearly 30% of the injected material is present in the blood after 24 hours.

2. 24 Hour Tissue Levels.

Studies to determine the distribution of gallium labelled liposomes in selected tissues, 24 hours after intravenous liposome injection, were carried out. The liposome composition (0.4 ml) was injected IV in animals. The percent dose remaining in tissues 24 hours after intravenous administration are shown in Table 1.

While the invention has been described with reference to specific methods and embodiments, it will be appreciated that various modifications and changes may be made without departing from the invention.

IT IS CLAIMED:

1. In a method of treating a subject by parenteral administration of an immunogenic polypeptide, the improvement comprising

pretreating the subject by parenteral administration of a polymer-polypeptide composition containing the polypeptide and polyethylene glycol (PEG) chains which surround, but are not covelently attached to, said polypeptide.

2. The method of claim 1, wherein the polypeptide is partially surrounded by relatively long PEG chains which form a polymer chain layer on a particle surface, and the polypeptide is attached to the particle surface.

3. The method of claim 2, wherein the particle surface is the outer surface of a liposome.

4. The method of claim 3, wherein the PEG chains have sizes between about 1,000-10,000 daltons.

5. The method of claim 4, wherein the immunogenic polypeptide is a xenogeneic antibody or antibody F_{ab} fragment.

6. The method of claim 5, wherein the antibody or antibody F_{ab} is attached to liposome surface by high affinity, specific antibody binding to an antigen substrate covalently attached to the liposome surface.

7. The method of claim 4, for use in treating a subject to reduce an allergic response to a polypeptide allergen, wherein the immunogenic polypeptide is an allergen attached to the liposome surface by biotin/avidin coupling.

8. The method of claim 4, wherein the immunogenic polypeptide is an allergen attached directly to a lipid polar head group on said liposome surface.

9. The method of claim 4, wherein the polypeptide is an immunogenic enzyme attached to the liposome surface by high affinity binding to an inhibitor substrate.

10. A composition for use in pretreating a subject before parenteral administration of a therapeutic, immunogenic polypeptide which is reactive with a selected substrate, comprising

a particle support having surface-bound molecules of said substrate,

a layer of polyethylene glycol (PEG) chains carried on the outer surface of the support, and

the polypeptide, anchored to said support by binding to said substrate, wherein the polypeptide is surrounded by, but not covalently attached to, said PEG chains.

11. The compositon of claim 10, wherein said layer is composed of PEG chains having sizes between about 1,000-10,000 daltons.

12. The composition of claim 10, wherein the particle support is a liposomal particle, and the substrate is directly attached to the liposome surface.

13. The composition of claim 10, wherein the substrate is an antigen, and the polypeptide is an antibody or antibody F_{ab} fragment.

14. The composition of claim 10, wherein the substrate is an enzyme inhibitor, and the polypeptide is an enzyme having a high affinity binding site for said inhibitor.

15. A composition for use in treating a subject to reduce an allergic response to a polypeptide allergen, comprising

a particle support,

a layer of polyethylene glycol (PEG) chains carried on the outer surface of the support, and

the polypeptide allergen anchored to said support to the surface thereof, wherein said allergen is surrounded by, but not covalently attached to, said PEG chains.

16. The composition of claim 15, wherein said layer is composed of PEG chains having sizes between about 1,000-10,000 daltons.

17. The composition of claim 15, wherein the particle support is a liposomal particle.

18. The composition of claim 16, wherein the allergen is directly attached to the liposomal particle.

19. The composition of claim 17, wherein the allergen is anchored to the liposomal particle by biotin/avidin coupling.

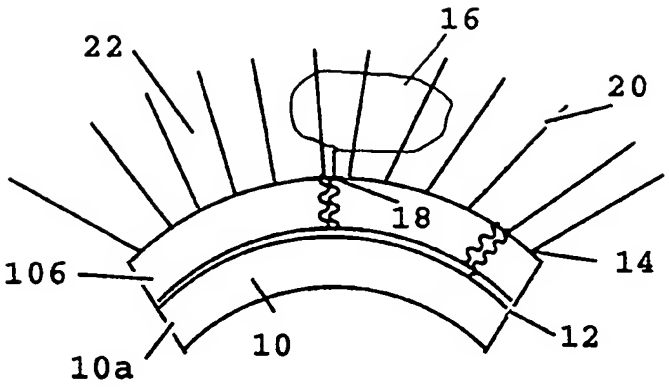


Fig. 1A

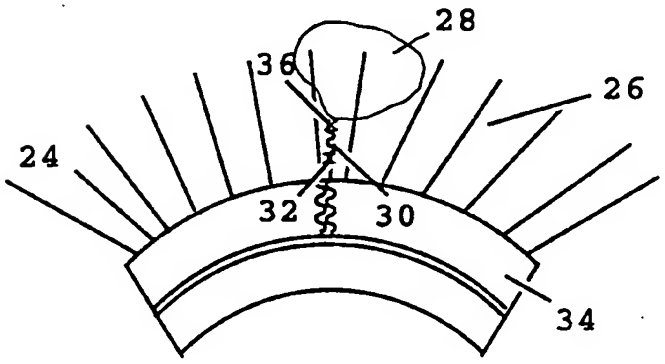


Fig. 1B

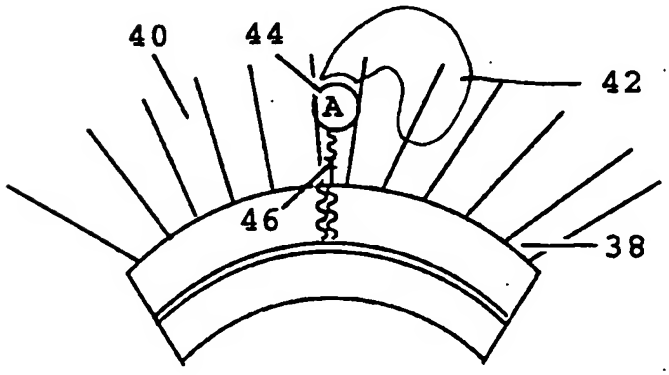


Fig. 1C

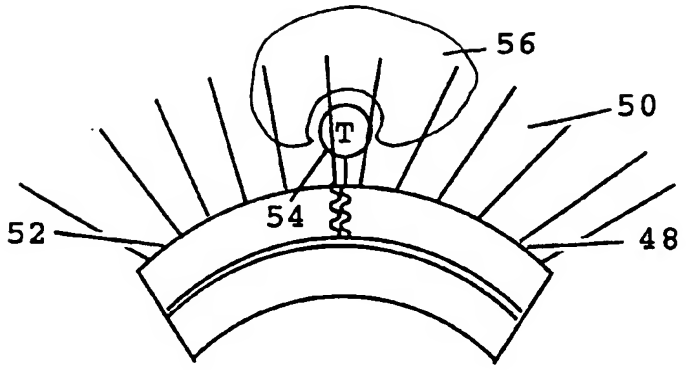


Fig. 1D

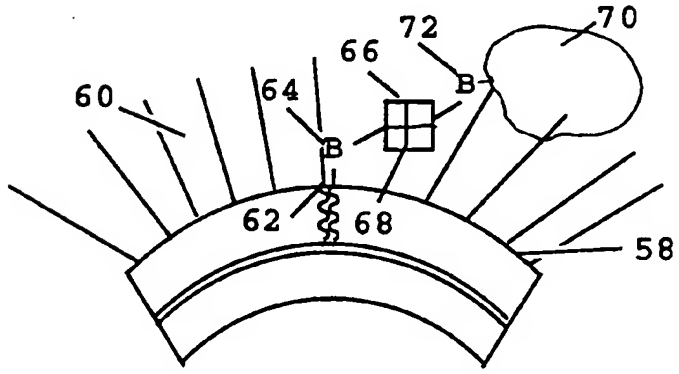


Fig. 1E

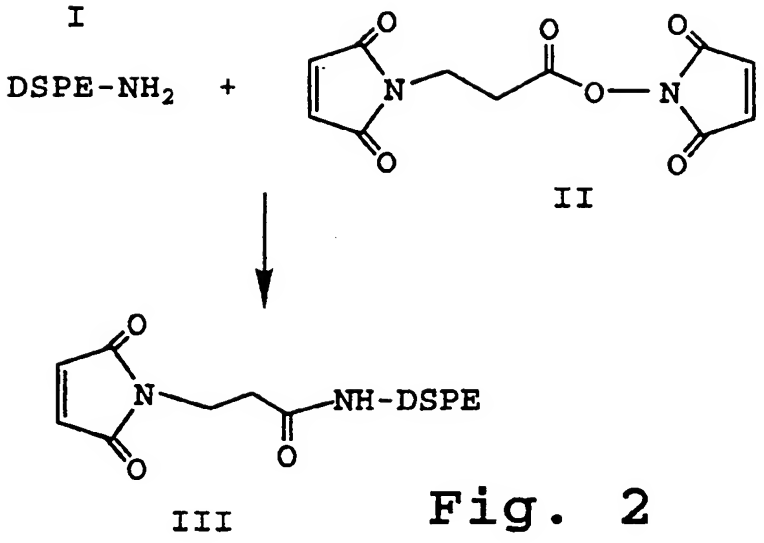


Fig. 2

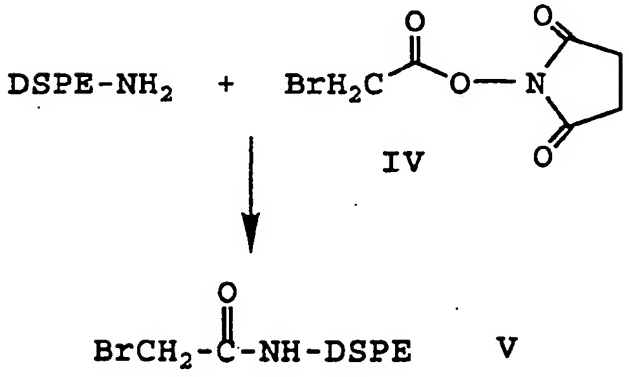


Fig. 3

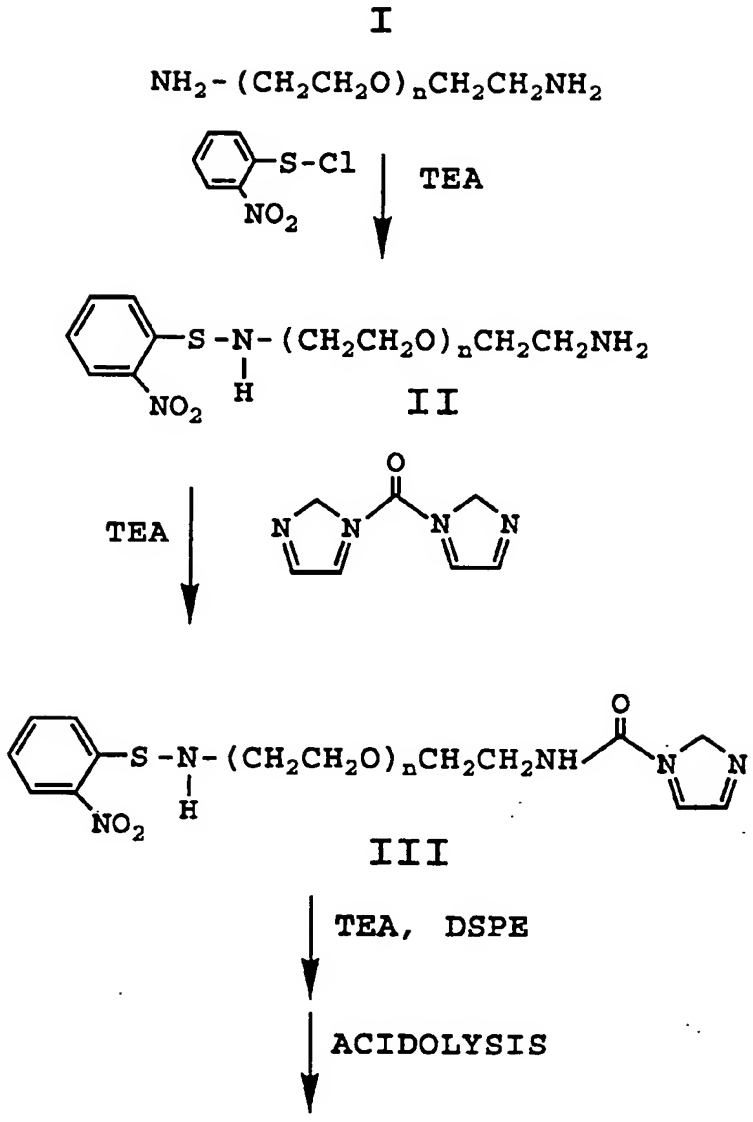

I
NH2-(CH2CH2O)nCH2CH2NH2
S-Cl
NO2
TEA
S-N-(CH2CH2O)nCH2CH2NH2
H
NO2
II
TEA
O
N
N
N
N
S-N-(CH2CH2O)nCH2CH2NH
H
NO2
O
N
N
III
TEA, DSPE
ACIDOLYSIS

Fig. 4

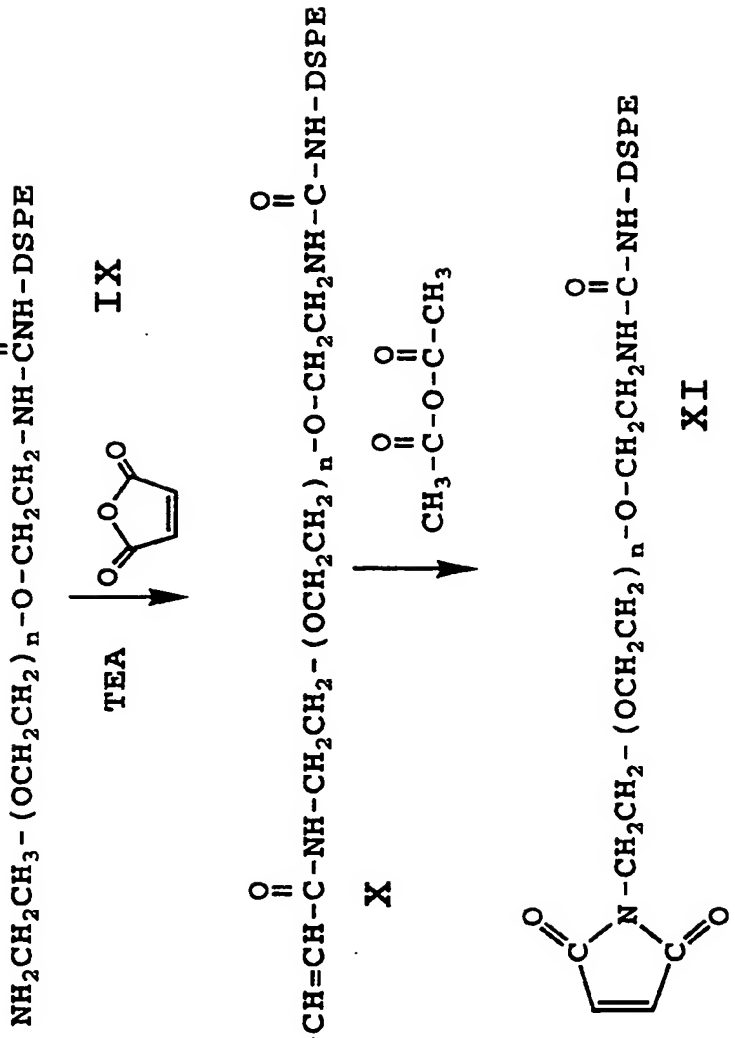

NH2CH2CH3-(OCH2CH2)n-O-CH2CH2-NH-C-NH-DSPE
IX
TEA
-O-C-CH=CH-C-NH-CH2CH2-(OCH2CH2)n-O-CH2CH2NH-C-NH-DSPE
X
CH3-C-O-C-CH3
N-CH2CH2-(OCH2CH2)n-O-CH2CH2NH-C-NH-DSPE
XI

Fig. 4 (con't)

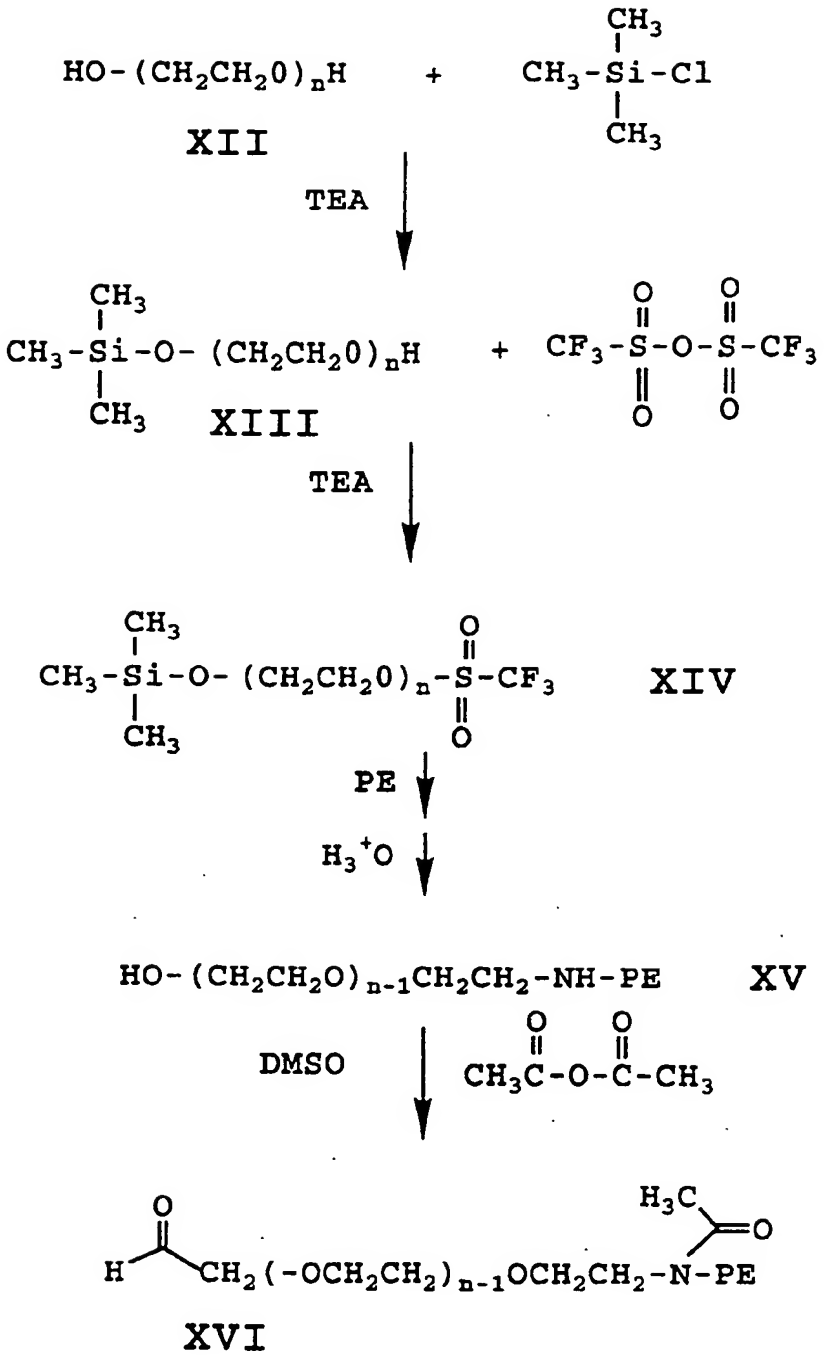
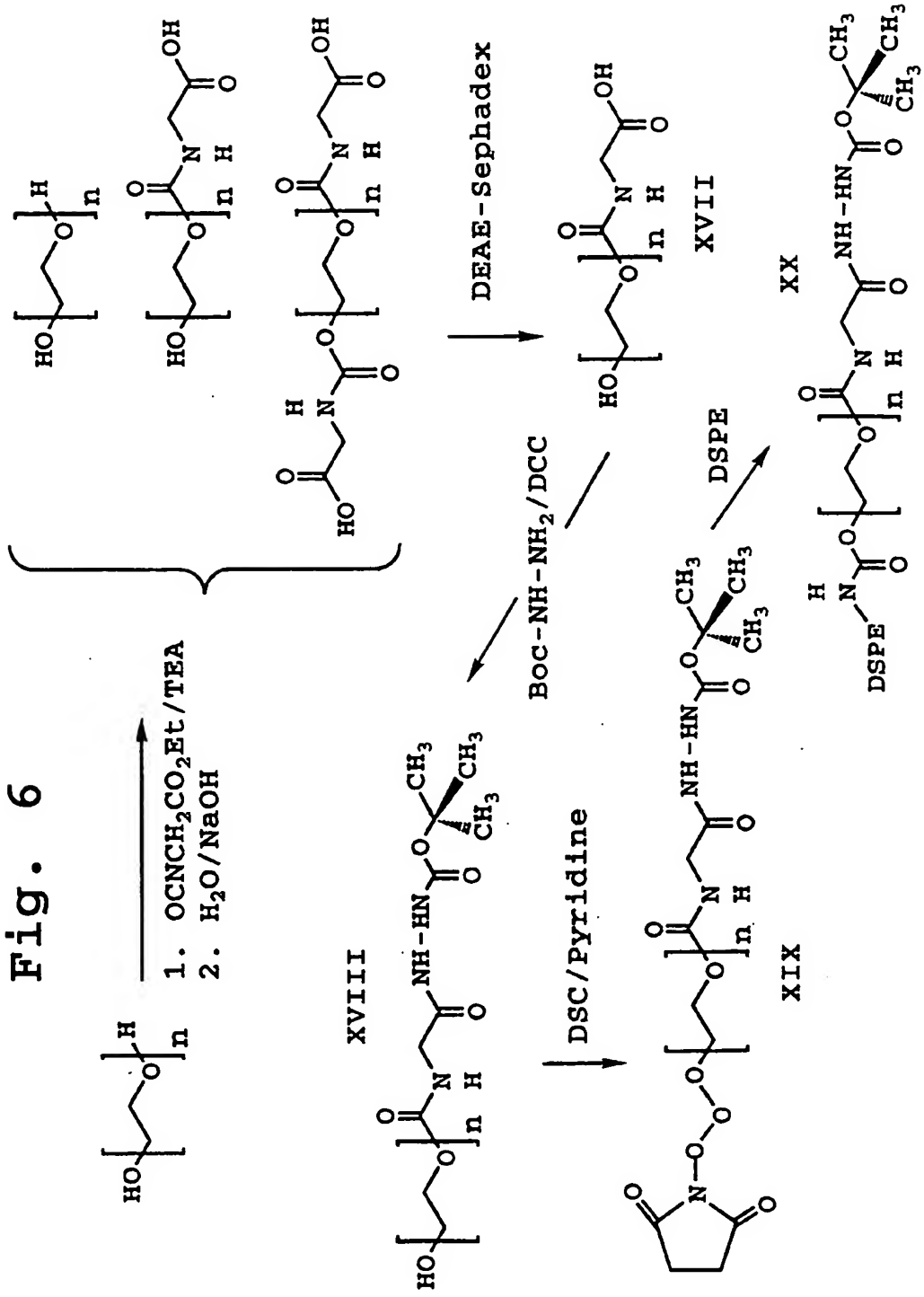

HO-(CH2CH20)nH
+
CH3-Si(CH3)2-Cl
XII
TEA
CH3-Si(CH3)2-O-(CH2CH20)nH
+
CF3-S(=O)2-O-S(=O)2-CF3
XIII
TEA
CH3-Si(CH3)2-O-(CH2CH20)n-S(=O)2-CF3
XIV
PE
H3+O
HO-(CH2CH2O)n-1CH2CH2-NH-PE
XV
DMSO
CH3C(=O)-O-C(=O)-CH3
H-C(=O)-CH2(-OCH2CH2)n-1OCH2CH2-N(-PE)-C(=O)-CH3
XVI

Fig. 5

Fig. 6


1. $OCNCH_2CO_2Et$/TEA
2. H_2O/NaOH
DEAE-Sephadex
Boc-NH-NH_2/DCC
DSC/Pyridine
DSPE
XVIII
XVII
XIX
XX
DSPE

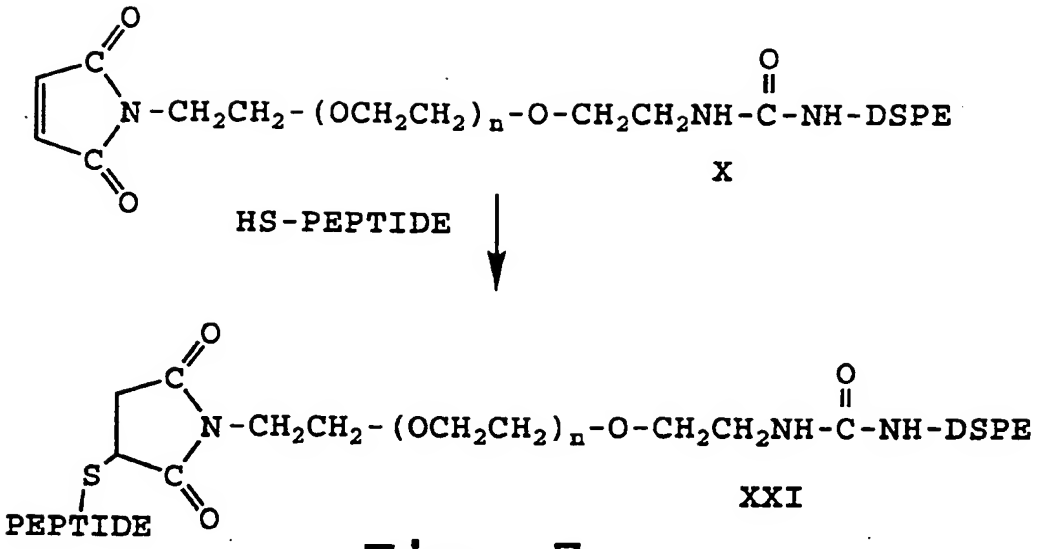

N-CH2CH2-(OCH2CH2)n-O-CH2CH2NH-C-NH-DSPE
X
HS-PEPTIDE
N-CH2CH2-(OCH2CH2)n-O-CH2CH2NH-C-NH-DSPE
S
PEPTIDE
XXI

Fig. 7

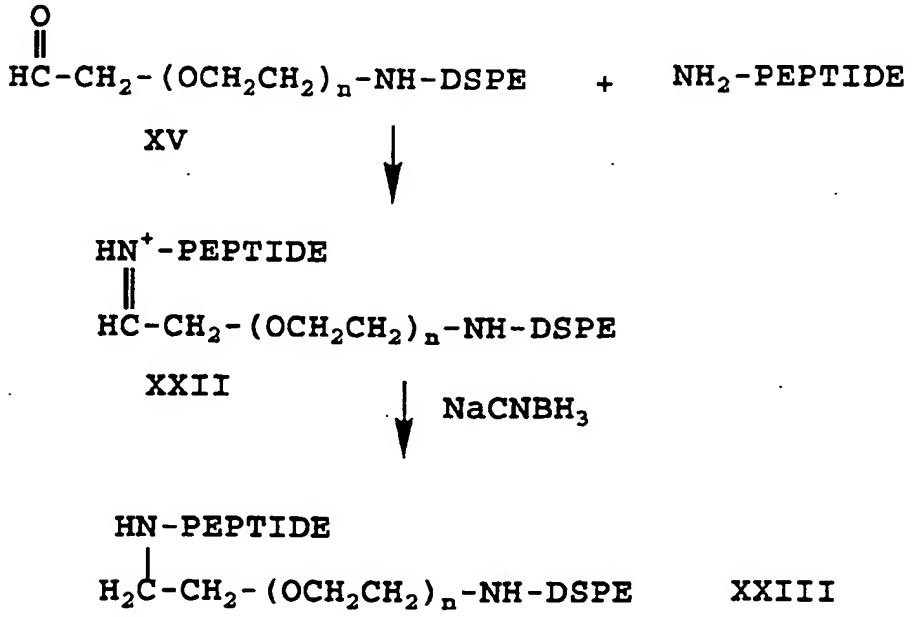

HC-CH2-(OCH2CH2)n-NH-DSPE
+
NH2-PEPTIDE
XV
HN+-PEPTIDE
HC-CH2-(OCH2CH2)n-NH-DSPE
XXII
NaCNBH3
HN-PEPTIDE
H2C-CH2-(OCH2CH2)n-NH-DSPE
XXIII

Fig. 8

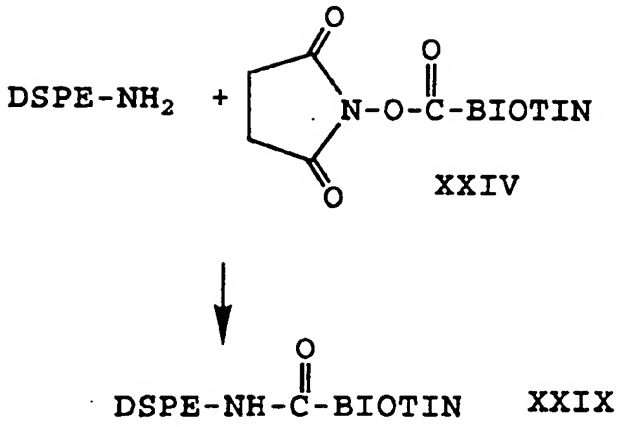


Fig. 9A

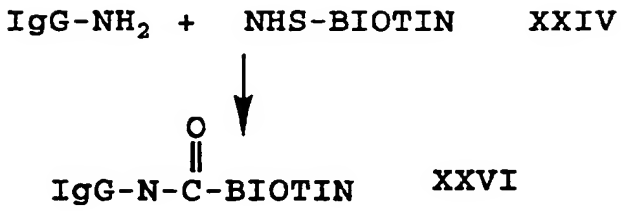


Fig. 9B

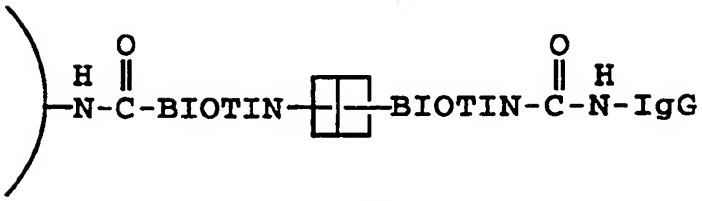


Fig. 9C

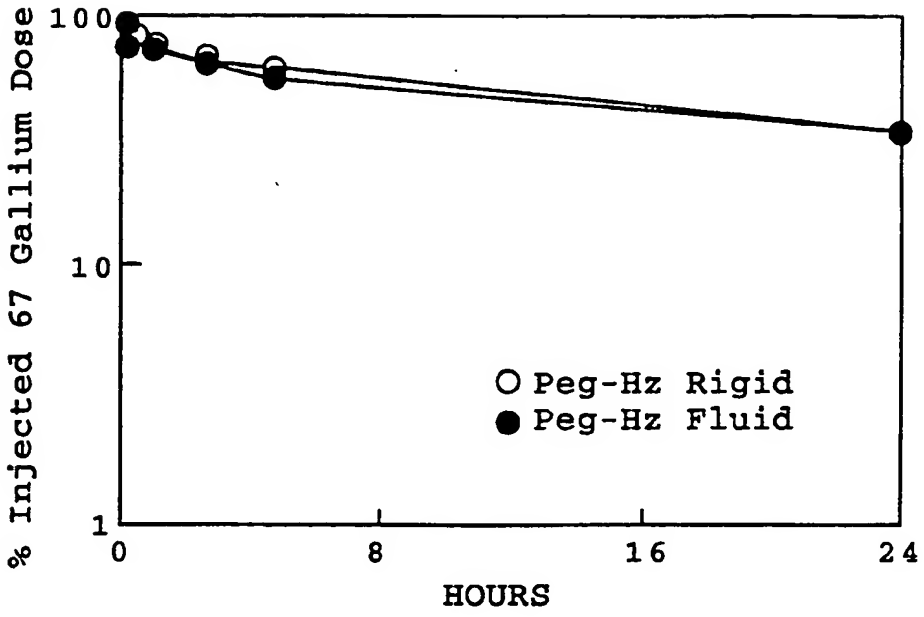

% Injected 67 Gallium Dose
100
10
1
0
8
16
24
HOURS
O Peg-Hz Rigid
● Peg-Hz Fluid

Fig. 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/03102

A. CLASSIFICATION OF SUBJECT MATTER
IPC(5) : A61K 37/02, 37/22, 37/48, 39/35, 39/395
US CL : 424/85.8, 91, 450; 514/2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/85.8, 91, 450; 514/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS ONLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,261,973 (LEE ET AL) 14 April 1981, col. 1 and col. 2.	1-19
Y	US, A, 5,013,556 (WOODLE ET AL) 07 May 1991, col. 12, lines 64-65 and the Abstract.	1-19
Y	US, A, 5,047,245 (BALLY ET AL) 10 September 1991, col. 1 and col. 2.	1-19
Y	US, A, 5,158,880 (EVELEIGH), 27 October 1992, col. 1, lines 23-25.	1-6, 9-14

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 MAY 1994	JUN 24 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer CHRISTINA CHAN Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/03102

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Int. J. Cancer, Supplement Volume 3, issued 1988, P. K. Maiti et al, "Tolerogenic Conjugates of Xenogeneic Monoclonal Antibodies with Monomethoxypolyethylene Glycol. I. Induction of Long-Lasting Tolerance to Xenogeneic Monoclonal Antibodies", pages 17-22, especially page 17.	1-6, 10-13
Y	The Journal of Biological Chemistry, Volume 252, No. 11, issued 10 June 1977, A. Abuchowski et al, "Effect of Covalent Attachment of Polyethylene Glycol on Immunogenicity and Circulating Life of Bovine Liver Catalase", pages 3582-3586, especially page 3585, right column and page 3586.	1-19
Y	Biochimica et Biophysica Acta, Volume 839, issued 1985, V. T. Kung et al, Antibody-Bearing Liposomes Improve Agglutination of Latex Particles Used in Clinical Diagnostic Assays", pages 105-109, especially page 105.	1-6, 10-13